Docket No.: 050229-0267

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	:	Customer Number: 20277
Peter Anthony CROOKS, et al.	:	Confirmation Number: 5136
Application No.: 09/881,215	:	Group Art Unit: 1614
Filed: June 15, 2001	:	Examiner: Zohreh A. Fay

For: AGMATINE AND AGMATINE ANALOGS IN THE TREATMENT OF EPILEPSY, SEIZURE AND ELECTROCONVULSIVE DISORDERS

TRANSMITTAL OF APPEAL BRIEF

Mail Stop Appeal Brief
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Submitted herewith is Appellant's Appeal Brief in support of the Notice of Appeal filed March 20, 2007. Please charge the Appeal Brief fee of $250.00 to Deposit Account 500417.

A petition for a one-month extension of time under 37 C.F.R. 1.136 is hereby made. Please charge any shortage in fees due under 37 C.F.R. 1.17 and 41.20, and in connection with the filing of this paper, including extension of time fees, to Deposit Account 500417 and please credit any excess fees to such deposit account.

Respectfully submitted,

McDERMOTT WILL & EMERY LLP

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APPEAL BRIEF

Mail Stop Appeal Brief
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This Appeal Brief is submitted in support of the Notice of Appeal filed March 20, 2007, wherein Appellant appeals from the Primary Examiner's rejection of claims 5, 7, 9, 11 and 13-20.

Real Party In Interest

This application is assigned to University of Kentucky Research Foundation by assignment recorded on December 27, 2001, at Reel 012404, Frame 0240.

Related Appeals and Interferences

There are no related appeals and interferences associated with the above-referenced patent application.

Status of Claims

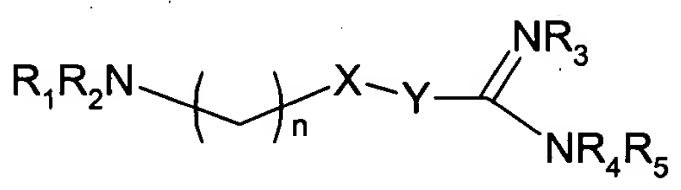
Claims 5, 7, 9, 11 and 13-20 are pending in this application and are under final rejection. Claims 1-4, 6, 8, 10 and 12 were previously cancelled and are no longer pending.

Status of Amendments

All amendments have been entered.

Summary of Claimed Subject Matter

Claims 5 and 12 are the only independent claims. Claim 5 is directed to a method of treating, ameliorating or preventing seizures associated with epilepsy by administering 0.1 to about 500 mg of an agmatine or an agamtine analog having the following formula:



wherein

n is 0 to about 10;

R_1, R_2, R_3, R_4, and R_5, are each independently, or any combination thereof: hydrogen, hydroxy, substituted or unsubstituted C_{1-10} alkyl, substituted or unsubstituted C_{3-8} cycloalkyl, substituted or unsubstituted arylalkyl (comprising Ar-$(CH_2)_m$; where Ar is aromatic and m is 0 to about 10) substituted or unsubstituted C_{1-10} alkoxyl, substituted or unsubstituted C_{1-10} acyl, halogeno, amido, phenyl, thio, or amino; and

X and Y are each independently: O, NH, CH_2, CF_2, Se, C=O, C=N, or C=S, or X-Y together is HC=CH, C≡C, N=N, N=CH, CH=N, or a saturated or unsaturated ring. Claim 13 is similar to claim 5 with the additional step of identifying a human subject in need of said treatment or prevention.

Claim 7 is dependent on independent claim 5 and further limits method claim 5 to a pharmaceutical composition comprising agmatine or its pharmaceutically acceptable salt and a pharmaceutically acceptable carrier.

Claim 9 further limits claim 7 by limiting the dosage amount to between about 0.1 and about 50 mg/kg per day indefinitely or until seizures associated with epilepsy.

Claims 11 and 17 limit method claims 5 and 13, respectively, to preventing or reducing seizures associated with epileptic activity.

Claims .

Grounds of Rejection To Be Reviewed By Appeal

1. Claims 5, 7, 9, 11 and 13-20 stand rejected under 35 U.S.C. § 112, first paragraph, as not being enabling for preventing seizure using agmatine.

2. It appears that claims 5, 7, 9, 11 and 13-20 stand rejected under 35 U.S.C. § 103 as being unpatentable over Uzbay et al. ("Effects of agmatine on ethanol withdrawal syndrome in rats," *Behavioural Brain Research*, Vol. 107, pp. 153-159 (2000)) in view of Rajasekaran et al. ("Effect of acute and repeated administration of nitric oxide (NO) precursor L-arginine, NO donor, sodium nitroprusside and NO synthase inhibitor, N(omega)-L-arginie methyl ester on picrotoxin - rinduced seizures in rats," http://www.uclm.es/inabis 2000/posters/files/129/session,

© 1999-2000).[1] However, the references relied upon by the Examiner is unclear. The final rejection was a rejection of the claims over Rajasekaran et al. for the reasons set forth on pages 3 and 4 of the Office Action dated May 5, 2005. However, the rejection referred to in the May 5th Office Action is an obviousness rejection based the combination of Uzbay et al. and Rajasekaran et al. The confusing nature of the rejection was pointed out in a response submitted by Appellant on June 28, 2006 (see page 2). However, the Examiner never clarified the rejection in the final rejection. See page 2 of the Office Action dated September 20, 2006 where the Examiner makes the rejection relying on the reasons given in the May 5, 2005 Office Action, but does not identify the reference or references relied upon for the rejection.

Argument

The following arguments pertain to claims 5, 9, 11, 13 and 17. Claim 7 stands or falls with claim 5 while claims 14-16 and 18-20 stand or fall with claim 13.

Rejection Under 35 U.S.C. § 112

The Examiner rejected claims 5, 7, 9, 11 and 13-20 under 35 U.S.C. § 112, first paragraph, as not being enabling for preventing seizure using agmatine. The Examiner also appears to be asserting that the disclosed invention is not commensurate in scope with the claims. The Examiner relies on the Wands factors for her position. Appellant respectfully disagrees.

The test of enablement is whether one skilled in the art could make or use the claimed invention from the disclosures in the specification coupled with information known in the art without undue experimentation. *United States V. Telectronics, Inc.*, 857 F.2d 778, 785, 8

[1] According to the Examiner, the rejections under 35 U.S.C. § 103 are based on the reasons set forth on pages 3 and 4 of the non-final Office Action dated May 5, 2005. In that Action, the statutory basis for the rejection did not identify which section of 35 U.S.C. § 103 the Examiner was relying upon.

USPQ2d 1217, 1223 (Fed. Cir. 1988), *cert. denied*, 490 U.S. 1046 (1989); *In re Stephens*, 529 F.2d 1343, 1345, 188 USPQ 659, 661 (CCPA 1976). Determining enablement is a question of law based on underlying factual findings. *In re Vaeck*, 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991); *Atlas Powder Co. v. E.I. Du Pont De Nemours & Co.*, 750 F.2d 1569, 1573, 224 USPQ 409, 411 (Fed. Cir. 1984). The Examiner has considered the Wand's factors, i.e., the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art, and the breadth of the claims. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404, (Fed. Cir. 1988), citing with approval *Ex parte Forman*, 230 USPQ 526, 547 (Bd. Pat. App. & Int. 1986). However, the reasoning of the Examiner is based on conclusions and not cogent scientific reasoning.

For the nature of the invention, the Examiner merely recites that the "claims are drawn to a method of treating, ameliorating or preventing seizures using agmatine." Nothing else is presented as to why and how the nature of the invention from the teachings of the prior art would lend itself to undue experimentation.

The Examiner concludes that the "prior art does not recognize that the prevention of seizures is done easily." For this conclusion, the Examiner relies an article to Lance which allegedly shows "different types of seizures are treated with different agents" and that there "are no teaching directed to prevention of seizures." The Lance was not cited by the Examiner in a PTO-892 as prior art. Nor did Appellant cite it in an Information Disclosure Statement. Moreover, the citation by the Examiner does not include a date. Therefore, Lance is not prior art

and it was improper for the Examiner to rely on it as prior art. Moreover, the conclusory statements by the Examiner do not show undue experimentation. The fact that different types of seizures are treated with different agents does not in and of itself indicated that a person having ordinary skill in the art would have to engage in experimentation that would be burdensome to practice the claimed subject matter.

As for the level of skill in the art, the Examiner concludes that it is "high." No evidence has been presented by the Examiner to establish this fact. The Examiner has not presented any evidence to establish that the level of skill in treating seizures is so extraordinary and difficult, that the level of skill should be regarded as "high." The Examiner has rendered her own opinion, and not a fact based on evidence.

The Examiner concludes that the "claims are very broad and encompass a composition for treating or preventing seizures using agmatine." This a conclusion or opinion of the Examiner. No evidence or cogent reasoning has been presented to establish why the claims are very broad. The meaning that the claims "encompass a composition for treating or preventing seizures using agmatine" is not understood. This is what Appellant is claiming. It is not clear how this would render the claims unduly broad in scope.

The Examiner asserts that Appellant's specification fails "to set forth a representative number of examples to demonstrate the effect of agmatine on preventing seizures." This fact alone does not establish undue experimentation. A patent specification is not a blue print and the patent statutes do not require the patent applicant to present multiple examples regarding the claimed subject matter. The statute requires applicant to provide a description in such a matter that one skilled in the art can make and use the invention.

Rejection under 35 U.S.C. § 103 over Uzbay et al.
in view of Rajasekaran et al. or over Rajasekaran alone

Claims 5, 7, 9, 11 and 13-20 appear to be rejected under 35 U.S.C. § 103(a) as being unpatentable over Uzbay et al. in view of Rajasekaran et al., but the claims may be rejected over Rajasekaran et al. alone. According to the reasons relied upon by the Examiner on pages 3 and 4 of the Office Action dated May 5, 2005, the Examiner finds that Uzbay teaches the use of 40 mg of agmatine to treat audiogenic seizure due to ethanol withdrawal while Rajasekaran et al. teaches that anticonvulsant activity of agmatine used in the treatment of seizure due to epilepsy and that the underlying mechanism of anticonvulsant activity is inhibiting NO production. From these teachings, the Examiner concludes that it would have been within the skill of the art to modify Uzbay to treat seizures cause by epilepsy. The Examiner has not established a *prima facie* case of obviousness.

The teachings of Uzbay *et al.* are clearly limited to and relevant only to ethanol withdrawal syndrome associated. In particular, this reference teaches at page 155, §3.3 that agmatine "reduced, dose-dependently and significantly, the intensity of stereotyped behavior and incidence of wet dog shakes and tremors appearing during the ethanol withdrawal." However, the reference goes on to disclose agmatine "reduced both incidence and intensity of the audiogenic seizure appearing at the 6th h. of ethanol withdrawal, dose-dependently (Table 2), but the inhibitory effect of agmatine did not reach a statistically significant level" (underscoring added for emphasis). Clearly, these authors did not conclude that agmatine is useful for treatment of audiogenic seizures as the Examiner asserts. These authors conclude that agmatine

is useful for treating the syndrome of ethanol withdrawal, but that it has no significant effect on seizures associated with ethanol withdrawal.

The Examiner asserts that Uzbay et al. teach "that the therapeutic effects [of agmatine] are resulted from blocking nitric oxide synthesis and selective inhibition of the NMD subclass of glutamate receptor channels." However, the authors actually state that the inhibitory effects of agmatine on ethanol withdrawal syndrome may be explained by any of three different mechanisms. They do not speculate as to which of the various mechanisms come into play in the treatment of ethanol withdrawal syndrome. See page 156. Contrary to the Examiner's assertions, these authors did not conclude that the therapeutic effect of agmatine on ethanol withdrawal syndrome is through inhibition of the NMD subclass of glutamate receptor channels. Certainly, they did not speculate about mechanisms of action concerning the effects of agmatine on seizures associated with ethanol withdrawal syndrome, since their data show that there was no statistically significant effect on seizures.

The data in Uzbay et al. establish a failed attempt to treat seizures with agamatine, i.e., these studies agmatine was not effective in treating seizures related to alcohol abuse. Moreover, they do not suggest that agmatine is a useful treatment for seizures in general. Clearly, the reference teaches away from the use of agmatine to treat alcohol-related seizures, and more particularly, seizures in general. Therefore, the reference cannot possibly be construed to suggest the use of agmatine to treat seizures associated with epilepsy. On this basis alone, the skilled practitioner would not have been motivated to use agmatine for treatment of seizures associated with epilepsy.

Rajasekaran et al. taken in combination with Uzbay et al. do not cure the deficiencies of Uzbay et al. Rajasekaran et al. merely suggest that the anticonvulsant activity of L-arg may be effected through agmatine, but they offer no evidence to that effect. Rajasekaran et al. can be interpreted, at best, as a suggestion to try agmatine as an anticonvulsant, but there is no indicia of an expectation of success in the cited reference, as required under 35 U.S.C. § 103. Moreover, in view of the data set forth in Uzbay et al., the combination of prior art suggests that anticonvulsive activity is not effected through agmatine. Thus, the combination of prior art cited by the Examiner is not seen as establishing a *prima facie* case of obviousness.

As for Rajasekaran et al. taken alone, the Examiner states that Rajasekaran et al., "teaches the anticonvulsant activity of agmatine used in the treatment of seizure due to epilepsy" and teaches that the underlying mechanism for anticonvulsant activity is "utilizing NO inhibition, where NO is produced in the neurons in response to activation by NMD receptors." However, Rajasekaran et al. do not address the effects of agmatine on convulsions, but instead tested only the L-arginine (L-arg). Under the "Discussion" portion on the 3rd page of the reference, the reference states: "The anticonvulsant activity of [L-arginine] may be the direct ..., or a product of its metabolism such as agmatine (Li et al., 1995) or to the possible accumulation of L-arg per se ...." In referring to agmatine, Rajasekaran et al. teach that the anticonvulsant activity is a "product of its metabolism." Appellant understands this to mean that the metabolism mechanism of L-arginine, and not agmatine itself, causes anticonvulsant activity. The Li et al. article referenced in the Rajasekaran disclosure is directed to the metabolism of arginine to form agmatine. A copy of the Li et al. article is attached in the Evidence Appendix. There is no disclosure in Li et al. that agmatine alone exhibits anticonvulsant activity. From the disclosure

of Li et al, the conclusion by Rajasekaran et al. regarding agmatine is speculative at best. In addition, Rajasekaran et al. do not provide any data concerning agmatine to show that it is effective for treating, ameliorating or preventing seizures associated with epilepsy as required by claims 5 and 13. Since there are no data in this reference demonstrating that agmatine is the active metabolite of L-argenine, this disclosure is merely a suggestion to try agmatine. The reference does not provide a reasonable expectation of success should agmatine be used to treat epilepsy, as is required under 35 U.S.C. § 103. *See Northern Telecom Inc. v. Datapoint Corp.*, 15 USPQ2d 1321, 1323 (Fed. Cir. 1990); *In re Dow Chemical Co.*, 837 F.2d .469, 5 USPQ1529 (Fed. Cir. 1988).

Neither Uzbay et al. nor Rajasekaran et al. provide any evidence that the proposed NO inhibiting mechanism is indeed valid. In the abstract to the Rajasekaran et al. reference, the authors state that "The role of nitric oxide (NO) in seizures remain debated," yet the examiner asserts that the "known effects" of agmatine on NO synthesis render the claimed invention obvious. Further, Uzbay et al. do not provide the mechanism of action of agmatine as asserted by the Examiner, but merely suggest several possibilities, one of them being the NO inhibiting mechanism. However, regardless of which of the various proposed mechanisms of actions of the drug proffered by the authors is correct (if any), the data presented in Uzbay et al. clearly show that agmatine is not effective in treating seizures and one of ordinary skill in the art would recognize from the authors' conclusion that agmatine is not an effective treatment for seizures.

The confusion with regard to the cause of seizures and the conclusions drawn by Rajasekaran et al. makes it abundantly clear that there is nothing predictable or obvious about the treatment of seizures. Rajasekaran et al. merely speculate as to causes and possible treatments for

seizures, and this speculation amounts to nothing more than a suggestion to try any of the treatments proposed in this non-peer review poster.

During prosecution, Appellant cited Del-Bel et al., reference that demonstrates that "the effects of NOS inhibitors vary with the model of seizure." The reference is attached in the Evidence Appendix. Thus, the Examiner's reliance on proposed mechanisms of actions, and selection of a single mechanism from three possibilities proposed by Uzbay et al. reference is misplaced and does not demonstrate obviousness of the claimed invention. If Uzbay et al. and Rajasekaran et al. are not certain of the mechanism of action of agmatine, it is unclear how the Examiner can be certain.

Rajasekaran et al. do not suggest that any one of the speculative treatments may be more successful than the others. Significantly, there are no data in this reference that suggest that administration of agmatine will successfully treat or prevent seizures associated with epilepsy. Thus, this is further evidence that the combined teachings of Rajasekaran et al. and Uzbay et al. would not have led a person skilled in the art to the present invention. Instead, this combination of prior art clearly shows that at the time of the invention, little was known about the cause of seizures, and even less about treatment of seizures. Further Uzbay et al. teach away from the use of agmatine to treat seizures. In order to establish a *prima facie* case of obviousness, the Examiner must show that the prior art teaches a reasonable expectation of success of the claimed method. *Amgen, Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 1207-08. The prior art cited by the Examiner fails to show an expectation of success, and instead teaches that at the time of the invention, little was known as to the cause of seizures or how any treatment actually worked.

The Examiner's response to these arguments was to conclude that Appellant "alleges criticality to the lack of data in the prior art demonstrating the effectiveness of agmatine against seizures" and that the allegation is not well taken." The Examiner pointed to *In re Lambert*, 192 USPQ 278, 280 (CCPA 1976) as holding that the question of obviousness does not require absolute predictability. The Examiner has conceded the following, however, by not presenting any arguments to the contrary:

1. Uzbay et al. is limited to and relevant only to ethanol withdrawal syndrome and that it has no significant effect on seizures associated with ethanol withdrawal.

2. Rajasekaran et al. did not address the effects of agmatine on convulsions, but instead tested only tested L-arginine.

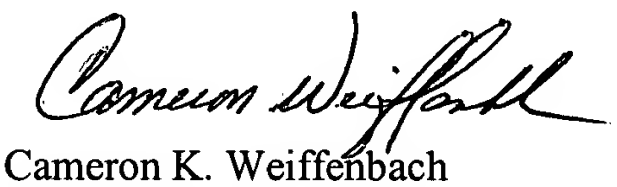
3. While Rajasekaran et al. conclude that the effects of L-arginine "may be direct, or a product of its metabolism such as argmatine ...", it is pure speculation and merely a suggestion to try agmatine.

Conclusion

For all of the foregoing reasons, Uzbay et al. and Rajasekaran et al., either taken alone or in combination do not render the claimed invention *prima facie* obvious. Appellant respectfully submits that the grounds of rejection of the claims on appeal is in error and should be reversed.

Respectfully submitted,

McDERMOTT, WILL & EMERY



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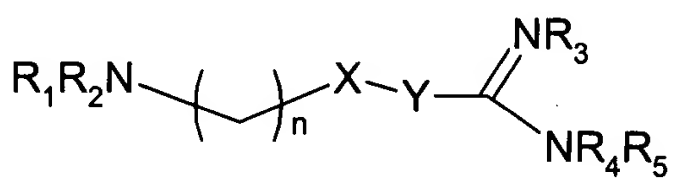
CLAIMS APPENDIX

5. A method of treating, ameliorating, or preventing seizures associated with epilepsy in a subject in need thereof, the method comprising:

administering a pharmaceutical composition comprising about 0.1 to about 500 mg of agmatine or an agmatine analog , or a pharmaceutically acceptable salt thereof per kilogram of the subject's weight to treat, reduce, or prevent seizures associated with epilepsy in the subject, wherein the agmatine analog has the following formula



wherein n is 0 to about 10;

R_1, R_2, R_3, R_4, and R_5, are each independently, or any combination thereof: hydrogen, hydroxy, substituted or unsubstituted $C_{1\text{-}10}$ alkyl, substituted or unsubstituted $C_{3\text{-}8}$ cycloalkyl, substituted or unsubstituted arylalkyl (comprising Ar-$(CH_2)_m$; where Ar is aromatic and m is 0 to about 10) substituted or unsubstituted $C_{1\text{-}10}$ alkoxyl, substituted or unsubstituted $C_{1\text{-}10}$ acyl, halogeno, amido, phenyl, thio, or amino; and

X and Y are each independently: O, NH, CH_2, CF_2, Se, C=O, C=N, or C=S, or X-Y together is HC=CH, C≡C, N=N, N=CH, CH=N, or a saturated or unsaturated ring.

7. A method according to claim 5, wherein the pharmaceutical composition comprises agmatine or its pharmaceutically acceptable salt and a pharmaceutically acceptable carrier.

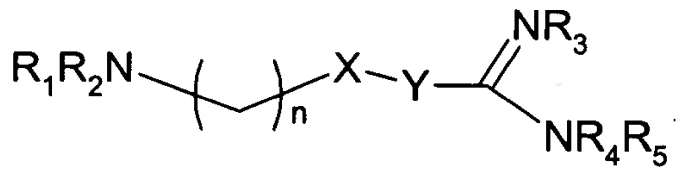
9. A method according to claim 7, wherein the composition is administered in a dose of about 0.1 to about 50 mg/kg per day indefinitely or until seizures associated with the epilepsy.

11. A method according to claim 5, comprising preventing or reducing seizure activity.

13. A method of treating or preventing seizures associated with epilepsy in a human comprising:

identifying a human subject in need of said treatment or prevention; and

administering about 0.1 to about 500 mg of agmatine or an agmatine analog , or a pharmaceutically acceptable salt thereof per kilogram of the subject's weight to the human subject, wherein the agmatine analog has the following formula



wherein n is 0 to about 10;

R_1, R_2, R_3, R_4, and R_5, are each independently, or any combination thereof: hydrogen, hydroxy, substituted or unsubstituted $C_{1\text{-}10}$ alkyl, substituted or unsubstituted $C_{3\text{-}8}$ cycloalkyl, substituted or unsubstituted arylalkyl (comprising Ar-$(CH_2)_m$; where Ar is aromatic and m is 0 to about 10) substituted or unsubstituted $C_{1\text{-}10}$ alkoxyl, substituted or unsubstituted $C_{1\text{-}10}$ acyl, halogeno, amido, phenyl, thio, or amino; and

X and Y are each independently: O, NH, CH_2, CF_2, Se, C=O, C=N, or C=S, or X-Y together is HC=CH, C≡C, N=N, N=CH, CH=N, or a saturated or unsaturated ring.

14. A method according to claim 13, comprising identifying a human subject in need of said treatment by analyzing an electroencephalogram taken of the human subject.

15. A method according to claim 13, comprising identifying a human subject in need of said treatment by observing the occurrence of a seizure in said subject.

16. A method according to claim 13, comprising administering the effective amount of agmatine, an agmatine analog or a pharmaceutically acceptable salt thereof to the human subject indefinitely or until the seizures associated with epilepsy cease.

17. A method according to claim 13, comprising preventing or reducing seizures associated with epileptic activity.

18. A method according to claim 13, comprising administering the effective amount of agmatine, an agmatine analog or a pharmaceutically acceptable salt thereof as a pharmaceutical composition.

19. A method according to claim 13, comprising administering the effective amount of agmatine, an agmatine analog or a pharmaceutically acceptable salt thereof parenterally.

20. A method according to claim 13, comprising administering the effective amount of agmatine, an agmatine analog or a pharmaceutically acceptable salt thereof orally.

EVIDENCE APPENDIX

Li et al., "Agmatine Is Synthesized by a Mitochondrial Arginine Decarboxylase in Rat Brain", Annals New York Academy of Sciences, 763, pp325-329, 1995; referred to on pages 2 and 3 of the response filed June 28, 2006.

Del-Bel et al., "Anticonvulsant and proconvulsant roles of nitric oxide in experimental models," *Brazilian Journal of Medical and Biological Research*, August 1997, Vol 30(8), pp 971-979; http:/www.scielo.br/scielo.php?scrip+sci_artrtext&pid=S0100-879X19970008000010; referred to on page 7 of the response filed November 7, 2005.

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Agmatine Is Synthesized by a Mitochondrial Arginine Decarboxylase in Rat Brain

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We recently discovered that agmatine (decarboxylated arginine) is contained in bovine brain.[1] Moreover, the amine, never before detected in mammals,[2] has properties of a clonidine-displacing substance (CDS); it binds with reasonable affinities (K_i: 1-4 μM) to α_2-adrenergic receptors and to all classes of imidazoline (I-) receptors.[1] In bacteria, fungi, some parasites, and marine animals,[2-5] agmatine is synthesized from arginine by a soluble enzyme, arginine decarboxylase (ADC). Our finding that ADC was expressed in rat brain[1] was the first demonstration of the enzyme in mammals. Moreover, it indicated that mammalian agmatine is a product of local biosynthesis and not attributable to dietary or enteric bacterial sources.

In *Escherichia coli* there are two soluble cytosolic isoforms: one is constitutive or biosynthetic,[6] the other inducible or biodegradative.[2] A third form of ADC which is membrane associated has been found in nematode *C. elegans*.[7] We sought to determine if mammalian brain ADC was similar to any of these other forms.

MATERIALS AND METHODS

Rat brains were homogenized in HEPES-sucrose buffer (5 mM HEPES, pH 7.4; 2 mM DTT; 0.5 mM PMSF; 0.2 mM EDTA) with a Teflon-glass homogenizer and centrifuged at 1,000 × *g* for 10 minutes. The pellet (P1) containing the nuclear fraction was saved for enzyme assays. The supernatant was centrifuged at 12,000 × *g* to produce a crude mitochondrial/synaptosomal pellet (P2). The resulting supernatant was centrifuged at 30,000 × *g* for 20 minutes generating a pellet (P3) containing the plasma/microsomal membrane fraction. This was used for enzyme assays. Separate mitochondrial or synaptosomal fractions were isolated from the P2 pellet by Percoll density gradients.[8] Membranes from each fraction were used for enzyme assays.

Except for studies on enzyme distribution in each subcellular compartment, ADC activity was measured in the P2 pellet which contained mitochondrial/synaptosomal membranes. Arginine decarboxylase was assayed by the method of Wu and Morris[9] measuring conversion of ^{14}C-arginine to $^{14}CO_2$. In brief, the tissue pellet was suspended in pre-chilled subcellular fractionation buffer without sucrose, homogenized by a Polytron (Brinkman, setting 6 for 2 × 15 s), and centrifuged at 100,000 × *g* for

15 minutes. The enzyme reaction was performed in glass tubes with a center well inserted into a tightly closed rubber stopper. The center wells contain strips of filter paper moistened with benzethionium or methylbenzethionium hydroxide to trap the $^{14}CO_2$ produced. The reaction mixture consisted of 500 µl of assay buffer: 10 mM Tris-HCl, pH 8.25, at 30°C; 1 mM DTT; 0.5 mM PMSF; 0.2 mM EDTA; 1 mM $MgSO_4$; 0.2 mM L-arginine and 0.4 µCi of L-[1-^{14}C]-arginine, specific activity 55 mCi/mmol. It was incubated at 30°C for 1 hour in a shaking water bath and the reaction terminated by the addition of 100 µl of 40% trichloroacetic acid injected through the rubber stopper. After further incubation for 20 minutes at 37°C, the filter paper strips were transferred to scintillation vials and radioactivity was determined by liquid scintillation counting. Verification of the product was obtained independently by demonstrating generation of [^{3}H]agmatine from [^{3}H]arginine by HPLC (data not shown).

Ornithine decarboxylase activity was measured by modification of a similar CO_2-trapping method[7] using 0.2 mM L-ornithine and L-[1-^{14}C]-ornithine (specific activity 55 mCi/mmol) as substrates. Protein was measured with a commercial protein assay kit (Bio-Rad) using bovine serum albumin (BSA) as a standard.

RESULTS

Stability. Arginine decarboxylase is labile. Hence, all enzyme activity was lost within a few hours in whole brain when stored on ice, whereas about 50% of activity was lost overnight when stored at 4°C as a membrane suspension. The enzyme was sensitive to detergents. At concentrations of 1%, Triton X-100, NP 40, or CHAPS totally abolished ADC activity, and even at 0.1% concentration, enzyme activity was substantially decreased.

Subcellular Distribution. The subcellular distribution of enzyme was measured after separation into nuclear, mitochondrial/synaptosomal, and plasma membrane fractions. The highest activity, 9.69 nmol CO_2/h/mg protein, or ~89% of total activity was restricted to the mitochondrial/synaptosomal membrane fraction. The activity of the plasma membrane (0.77 nmol. CO_2/h/mg protein) and the nuclear pellet (<0.48 nmol CO_2/h/mg protein) was ~7% and ~4% of total activity, respectively. After further fractionation of the mitochondrial/synaptosomal fraction 80% of total activity (88.67 nmol CO_2/h/mg protein) appeared in mitochondrial membranes. Much of the remaining 20% of ADC activity within the synaptosomal membrane (20.22 nmol CO_2/h/mg protein) was probably attributable to mitochondria, because these organelles are abundant in synaptosomes. Thus, ADC appears to be highly, if not exclusively, associated with mitochondrial membrane.

Kinetics. The activity of ADC was linear with respect to reaction time to 90 minutes and with protein concentrations from 0.5-40 mg/ml. Under optimal reaction conditions, the K_m for arginine, as determined by double-reciprocal (Lineweaver-Burk) plots, was 0.75 mM. The V_{max} was 2.22 nmol/h/mg protein.

pH and Temperature Optima. The optimal pH for brain ADC activity was 8.25, close to that of the biosynthetic ADC of *E. coli.*[10] By contrast to other forms of ADC, the temperature optimum was 30°C. At 37°C, the enzyme was only about one third as active as at 30°C.

TABLE 1.
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TABLE 1. Effect of Substrate Analogs and Polyamines on Arginine Decarboxylase Activity[a]

Compound	Relative Activity (%)
None	100
Amino acids:	
L-Ornithine	18
L-Lysine	59.8
L-Glutamine	79.4
L-Histidine	93.7
D-Arginine	94.7
D-Ornithine	92.3
DFMO	98.5
Polyamines:	
Spermine	18.8
Spermidine	29.4
Agmatine	66.5
Putrescine	66.6
Nitric oxide synthase inhibitors:	
N^G-nitroarginine	79.6
N^G-methylarginine	78.8

[a] The activity of ADC was measured in rat brain mitochondrial membrane for 1 hour at 37°C. Control activity was 88.6 nmol CO_2/h/mg protein. All compounds were tested at 1 mM against the substrate concentration of 0.2 mM arginine.

Inhibition by Substrate Analogs. To determine the effect of substrate analogs on ADC activity, several amino acids were tested for their ability to inhibit the enzyme. As shown in TABLE 1, although ornithine inhibited ADC activity by 82% at 1 mM concentration, lysine inhibited the enzyme by 40%. Therefore, rat brain ADC has properties similar to those of ADC of *C. elegans*[7] but not of *E. coli* and can be considered arginine/ornithine decarboxylase. However, difluoromethylornithine (DFMO), a universal and irreversible inhibitor for all forms of ornithine decarboxylase,[11] could not inhibit rat brain ADC, indicating the distinction between the two forms of the enzyme.

Because in bacteria agmatine is a precursor of polyamines,[12] the effect of several polyamines on ADC activity was examined. Arginine decarboxylase activity was inhibited, in rank order by: spermine > spermidine > agmatine > putrescine. At a concentration of 1 mM, which was fivefold higher than the substrate concentration (TABLE 1), spermidine and spermine blocked 81% and 70% activity, whereas agmatine and putrescine blocked only one third of the enzyme activity. Arginine decarboxylase was not inhibited by the nitric oxide synthase inhibitors N^G-nitroarginine and N^G-methylarginine.

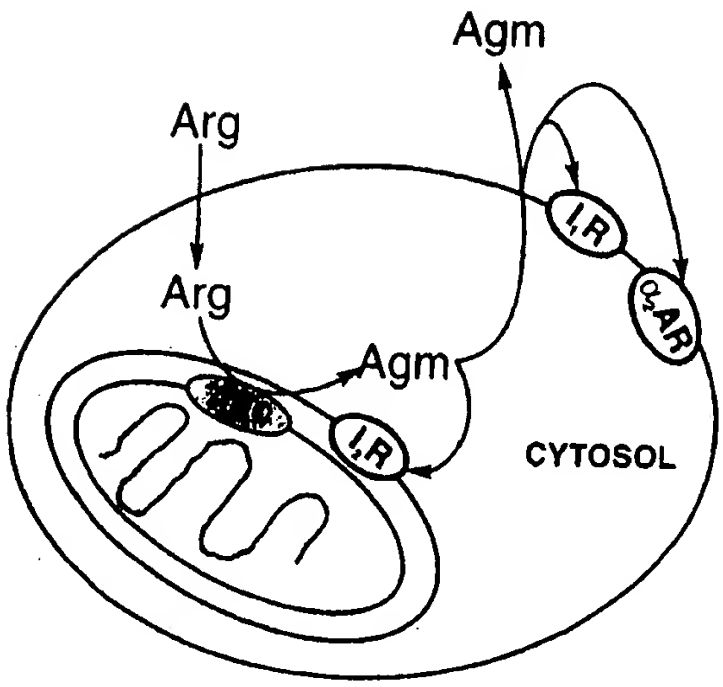

FIGURE 1. Schematic outline of the intracellular biosynthetic pathway for agmatine and its relationship to imidazoline (I-) and α_2-adrenergic receptors. See text for details.

DISCUSSION

This study confirms our previous observation that the rat brain expresses an enzyme that can synthesize agmatine and CO_2 from L-arginine and hence by definition represents an ADC.[1] The enzyme differs from the soluble bacterial forms in that it is associated with mitochondrial membranes. Like the constitutive form of *E. coli*,[10] it is optimally active at a high pH (8.25), but unlike that enzyme it does not require Mg^{2+} (unpublished observations).

Because rat brain ADC also uses ornithine as a substrate (K_m: 0.25 mM), it can be considered an arginine/ornithine decarboxylase. It differs, however, from ODC, a soluble cytoplasmic enzyme, which is irreversibly inhibited by DFMO. By contrast, DFMO is inactive against rat brain ADC. Rat brain ADC is most similar to a membrane-associated ODC isolated from the nematode C. elegans[7] which also uses arginine and ornithine as substrates; however, it is not known if the nematode enzyme is mitochondrial. However, the two enzymes also differ with respect to K_m, pH, and temperature optima and by the fact that the nematode enzyme is inhibited by DFMO.[7] Thus, rat brain ADC appears unique.

The localization of ADC to mitochondrial membranes is of special interest in view of the fact that imidazoline receptors of the I_2 subclass are also localized there.[13,14] FIGURE 1 schematically indicates the relation between the substrate, L-arginine, ADC, agmatine, and I- and α_2-adrenergic receptors in a typical cell. It emphasizes that the substrate L-arginine, which enters the cell by facilitated transport, is converted to agmatine on the mitochondrial membrane. Agmatine, so synthesized, can either bind to I_2 receptors on the mitochondrion, to α_2-adrenergic and possibly I_1 receptors on plasma membranes, or it can be released extracellularly, as evidenced by the presence of agmatine in serum.[14] Within the cell, agmatine may also regulate its own synthesis by feedback inhibition of ADC.

The data therefore suggests that ADC is a component of a complex and highly regulated system within cells generating agmatine, an amine that may act within and

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beyond the cell as an autocrine, paracrine, and hormonal messenger. We have argued[16] that agmatine may also be a novel neurotransmitter. If so, ADC undoubtedly participates in a potentially new neurotransmitter/neuromodulator network in the CNS.

CONCLUSIONS

1. Rat brain expresses ADC which differs from plant and bacterial forms by its localization to mitochondrial membranes and utilization of ornithine as well as arginine as substrate; yet, it is not a typical ODC as it is not cytosolic or inhibited by DFMO.

2. The colocalization of I_2 receptors and ADC to mitochondria suggests that agmatine may be an important intracellular message possibly regulating, through receptor mediation, its own biosynthesis. The presence of ADC and agmatine in mammalian brain raises questions of whether it might be an alternative pathway for polyamine biosynthesis in mammalian tissue.

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Anticonvulsant and proconvulsant roles of nitric oxide in experimental epilepsy models

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Abstract
Introduction
Material and Methods
Results
Discussion
References
Acknowledgments
Correspondence and Footnotes

Abstract

The effect of acute (120 mg/kg) and chronic (25 mg/kg, twice a day, for 4 days) intraperitonial injection of the nitric oxide (NO) synthase (NOS) inhibitor N^G-nitro-L-arginine (L-NOARG) was evaluated on seizure induction by drugs such as pilocarpine and pentylenetetrazole (PTZ) and by sound stimulation of audiogenic seizure-resistant (R) and audiogenic seizure-susceptible (S) rats. Seizures were elicited by a subconvulsant dose of pilocarpine (100 mg/kg) only after NOS inhibition. NOS inhibition also simultaneously potentiated the severity of PTZ-induced limbic seizures (60 mg/kg) and protected against PTZ-induced tonic seizures (80 mg/kg). The

audiogenic seizure susceptibility of S or R rats did not change after similar treatments. In conclusion, proconvulsant effects of NOS inhibition are suggested to occur in the pilocarpine model and in the limbic components of PTZ-induced seizures, while an anticonvulsant role is suggested for the tonic seizures induced by higher doses of PTZ, revealing inhibitor-specific interactions with convulsant dose and also confirming the hypothesis that the effects of NOS inhibitors vary with the model of seizure.

Key words: experimental epilepsy, seizures, forebrain, brainstem, pilocarpine, pentylenetetrazol, audiogenic seizures, genetically epilepsy-prone rats, GEPR, nitric oxide

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Introduction

Epilepsy is a phenomenon causing severe and continuous seizure activity such as that present in status epilepticus, or chronic spontaneous recurrent seizures. Alterations in several classic neurotransmitter systems such as the glutamatergic (1) or GABAergic (2) one have been implicated in the elicitation of epileptic seizures. More recently, other molecules such as nitric oxide (NO) have been pointed out as potential neurotransmitters or retrograde messengers (3) linked to synaptic plasticity (4) and regulation of brain excitability, including the triggering of seizure activity (5,6).

NO is formed from L-arginine by the enzyme NO synthase (NOS) (7) and the involvement of NO in epileptic disorders has been shown in experiments with systemic injection of NOS inhibitors (5,6,8,9). However, NOS inhibitor treatment has been reported either to augment (6,8,9) or to inhibit (6,10-13) experimentally induced seizures.

Mollace et al. (14) reported that L-arginine increased seizure severity in response to a subconvulsive dose of NMDA, suggesting that NO is a proconvulsant mediator. In contrast, during bicuculline-induced seizures, inhibition of NOS doubled the duration of the seizures (13,15). Inhibition of NOS attenuated kainate- (16) and tacrine-induced (17) convulsions, while in other studies NOS inhibition increased the severity of seizures and mortality during status epilepticus (12,18). Similar to the effect of NOS inhibitors during stroke, it is fair to assume that the discrepancies in the experimental findings may be due to the fact that the anti- or proconvulsant activity of NOS inhibitors is dose dependent, with lower doses affording protection (19). The alternative neurodestructive and neuroprotective roles of NO have been the subject of intense debate (reviewed by Choi (20); 21,22). The relative predominance of each effect has been ascribed to the physicochemical redox state of the NO molecule (23,24), to its tissue concentration (21) or to whether it was synthesized neuronally or in the cerebral vasculature (25,26). These factors might also influence the effects of NO on excitatory and inhibitory processes evoked by seizures, with varying results that might be difficult to predict.

The main goals of the present study were to obtain a better understanding of NO effects on seizures in order to determine if contradictory pro- and anticonvulsant results from the literature were due to the use of different epilepsy models. Assuming that these effects could be either neuroprotection or potentiation of seizures, we looked for seizure models that could show positive and negative effects. Pentylenetetrazole (PTZ)-evoked seizures can be either of the limbic type or generalized tonic-clonic seizures, depending on the dose of PTZ used. Additionally, pilocarpine (PILO) is able to induce acute limbic status epilepticus and complex partial type of seizures in the chronic state, when recurrent spontaneous seizures appear (27,28).

Subconvulsant PILO doses can be used in order to test the blocking effect of NOS on limbic seizure facilitation (9). Audiogenic genetically epilepsy-prone rats (GEPR-3s), Wistar and Sprague-Dawley resistant rats can be used to test seizure increase or *de novo* appearance after the treatments, whereas GEPR-3s and susceptible Wistar animals can be used to test the reduction in audiogenic seizure, which may suggest an involvement of NO in generalized tonic-clonic seizures (29,30). The seizure models currently used have common and distinct mechanisms. Limbic seizures are generally evoked by activation of forebrain excitatory mechanisms (31), while tonic-clonic seizures are related to GABA dysfunctions (PTZ) or to genetic (GABA and excitatory amino acids) alterations and sound sensitivity (audiogenic rats) (29,32).

Material and Methods

Animals

Adult male rats (200-300 g) were maintained on a constant 12/12-h light/dark cycle, with free access to food and water.

Group 1. Wistar rats received a subconvulsant dose of PILO (100 mg/kg, *ip*) and were treated acutely with either 0.9% saline (N = 7) or N^G-nitro-L-arginine (L-NOARG, *ip*) (N = 8).

Group 2. Wistar rats received a subconvulsant dose of PILO (100 mg/kg, *ip*) and were treated chronically with either 0.9% saline (N = 7) or L-NOARG (N = 6).

Group 3. Wistar rats received subconvulsant doses of PTZ (15 and 30 mg/kg, *ip*), or convulsant doses of PTZ (60 and 80 mg/kg, *ip*) and were treated acutely with L-NOARG (N = 8 for each dose).

Group 4. Wistar rats received subconvulsant doses of PTZ (15 and 30 mg/kg, *ip*), or convulsant doses of PTZ (60 and 80 mg/kg, *ip*) and were treated acutely with 0.9% saline (N = 8 for each subgroup).

Group 5. GEPR-3s and Sprague-Dawley control rats were submitted to acoustic stimulation, and treated acutely with either L-NOARG or 0.9% saline (N = 6 for each group).

Group 6. GEPR-3s and Sprague-Dawley control rats were submitted to acoustic stimulation and treated chronically with either L-NOARG or 0.9% saline (N = 6 for each group).

Group 7. Wistar audiogenic resistant rats were submitted to acoustic stimulation and treated acutely with either 0.9% saline (N = 8) or L-NOARG (N = 6).

Group 8. Wistar audiogenic resistant rats were submitted to acoustic stimulation and treated chronically with either 0.9% saline (N = 7) or L-NOARG (N = 8).

All experiments were performed in accordance with the rules of the Brazilian Society for Neurosciences and Behavior for animal experimentation.

Drugs

All drugs were purchased from Sigma Chemical Co. (St. Louis, MO). L-NOARG, PILO, methyl-scopolamine and PTZ were dissolved in sterile saline (0.9%). The systemic treatments were performed in a volume of 1 ml/kg body weight.

Treatments

Acute. Animals received L-NOARG (120 mg/kg, *ip*) or saline (0.9%) and 30 min later a dose of the convulsant drug (PILO or PTZ groups) or sound stimulation (audiogenic seizure (AS) groups).

Chronic. Animals were chronically treated with saline (0.9%) or L-NOARG (25 mg/kg, *ip*), twice a day for 4 days, and received a dose of one of the convulsant drugs or sound stimulation 30 min after the last injection.

Seizure models

Pilocarpine limbic seizures. Rats (groups 1 and 2) were injected with PILO nitrate (100 mg/kg, *ip*) or 380 mg/kg, *ip*, preceded by 1 mg/kg methyl-scopolamine in order to decrease peripheral cholinergic side effects.

PTZ-induced tonic-clonic seizures. Both subconvulsant (15 and 30 mg/kg, *ip*) and convulsant (60 and 80 mg/kg, *ip*) doses of PTZ were injected into adult Wistar rats.

Audiogenic seizures. Wistar and Sprague-Dawley resistant (R) animals, as well as susceptible (S) GEPR-3s were exposed to high-intensity (120 dB) acoustic stimulation for a maximum of 1 min or until tonic-clonic seizures appeared. Latencies and seizure severity indexes were then calculated.

Behavioral evaluation of seizure activity

Limbic seizures. To score limbic patterns such as those evoked by PILO or PTZ, we used the Racine (33) scale (classes 0 to 5) of limbic seizures as follows: 0 = immobility, 1 = facial automatisms, 2 = head nodding, 3 = unilateral forelimb clonus/bilateral forelimb clonus, 4 = bilateral forelimb clonus and rearing, and 5 = rearing, falling and generalized convulsions.

Audiogenic seizures. For the evaluation of audiogenic seizures (AS) in Wistar rats, an audiogenic severity index (SI), including a graded linear scale with a range of severity from SI = 0 (resistant rats) to SI = 1.0 (maximum, susceptible rats), was calculated (30). In order to determine the audiogenic susceptibility of the animals, three acoustic stimulations were performed before the beginning of the experimental protocol. For the Wistar animals, the most frequent behavioral sequences gave SI of the following values, which are derived from an audiogenic severity index we developed (30,34): 1) wild running (one fit) (SI = 0.11); 2) wild running with atonic falling and jumping during the running fits (SI = 0.23); 3) wild running (two fits), atonic falling, and jumping (SI = 0.38); 4) all the above plus tonic seizures (TCV; back arching tonus) (SI = 0.61); 5) all the above plus partial (only forelegs or hindlegs) and generalized (forelegs and hindlegs) clonic seizures (SI = 0.73); 6) all the above plus clonic spasms (SI = 0.85); 7) all the above plus head ventral flexion (SI = 0.9); 8) all the above plus forelimb hyperextension (SI = 0.95), and 9) all the above plus hindlimb hyperextension (SI = 1.0). The same SI was used for the highest PTZ doses because the induced seizures usually ended with wild running followed by tonic hyperextensions. For the GEPR-3s, which are

derived from Sprague-Dawley colonies, seizure severity was calculated using the audiogenic response scores (ARS) (0 to 9) described by Jobe et al. (29).

In the chemical models, latencies to the beginning of the seizure activity, and frequency of a given behavior were evaluated for 1 h. We mainly recorded the presence of piloerection, salivation, facial automatisms, myoclonus and status epilepticus, evaluated only by visual inspection. These behaviors have been widely described in previous experiments of the PILO model (27,28,35). In the case of audiogenic seizures, latency to running and to convulsion were also evaluated when present.

Statistical analysis

Incidence (%) of a given behavior was evaluated by the Fisher exact test ($P<0.05$). The values of the Racine scale or SI and ARS before and after treatments were compared by MANOVA.

Results

Pilocarpine (380 mg/kg, *ip*) elicited status epilepticus, followed by animal death (data not shown). No treatment was used (valium or thionembutal) to favor animal survival after status epilepticus because our main goal was to study only seizures induced by a high dose of PILO and by acute and chronic low doses of PILO. For this reason, all the animals treated with 380 mg/kg PILO died or were sacrificed after treatment. We did not intend to save the animals for spontaneous recurrent seizures.

A subconvulsant dose of PILO (100 mg/kg) induced behavioral modifications such as salivation, piloerection and facial automatisms, although this dose was not sufficient to elicit status epilepticus (0/14 animals; Figure 1). Acute pretreatment with L-NOARG of PILO-injected rats (100 mg/kg) strongly increased salivation (Figure 1A), piloerection (Figure 1B), and facial automatisms ($P<0.05$, Fisher exact test; Figure 1C). These effects were less pronounced in the chronically L-NOARG-treated group (Figure 1A, $P<0.05$; 1B and 1C, $P<0.01$). Status epilepticus was induced by PILO (100 mg/kg) in both the acutely and chronically L-NOARG-treated groups (Figure 1D), with a significant effect being observed in the acutely treated group ($P<0.05$). It is important to note that acute or chronic saline or L-NOARG treatment alone did not induce any seizures (groups 1 and 2) before PILO or any other treatment.

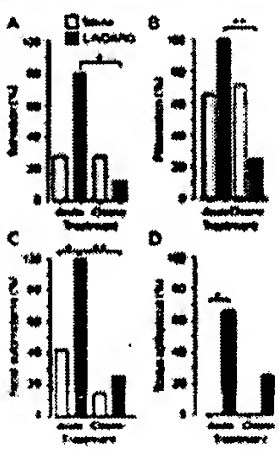

Figure 1 - Proconvulsant effects of L-NOARG on a subconvulsant pilocarpine (100 mg/kg, *ip*) dose. Observe the incidence of salivation (A), piloerection (B), facial automatisms (C) and status epilepticus (D) after acute or chronic L-NOARG treatment (*$P<0.05$; **$P<0.001$; Fisher exact test).

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Class 4-5 limbic seizures (Racine scale) were induced by a subconvulsant PILO dose (100 mg/kg) after both acute and chronic L-NOARG treatment ($P<0.01$, MANOVA; Figure 2). Observe in the same figure that, surprisingly, PILO alone also induced partial myoclonus (class 1-2; $P<0.05$) in the acute but not in the chronic group.

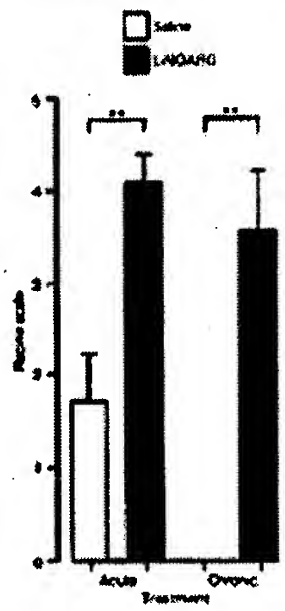

Figure 2 - Proconvulsant effects of acute or chronic L-NOARG treatment on a subconvulsant dose of pilocarpine (100 mg/kg, *ip*). Animals were evaluated for limbic seizures by the Racine scale (*$P<0.05$; **$P<0.01$; MANOVA).

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Behavioral alterations were not observed in rats submitted to NOS blockade and treated with the subconvulsant PTZ doses (15 and 30 mg/kg, Figure 3). However, the PTZ dose of 60 mg/kg induced class 4-5 limbic seizures (Figure 3), which were significantly worsened by acute L-NOARG treatment ($P<0.01$; MANOVA). The 80 mg/kg PTZ dose, a lethal dose, produced myoclonic jerks, generalized clonic seizures and tonic generalized extension, followed by animal death. Previous L-NOARG treatment significantly suppressed the incidence of tonic seizures and status epilepticus (Figure 4), and the severity of tonic seizures (Figure 5). In summary, these results demonstrate that NOS inhibition can exert both anticonvulsant and proconvulsant effects in the same epilepsy model (PTZ), depending on the convulsant dose used.

Audiogenic seizure-resistant rats (both Wistar and Sprague-Dawley) did not modify their behavior after L-NOARG-induced systemic NOS blockade and further acoustic stimulation (data not shown). Also, GEPR-3s, which are susceptible to audiogenic seizures, did not present changes in severity indexes or mean latencies to wild running or tonic-clonic seizure after acute or chronic L-NOARG treatment (data not shown).

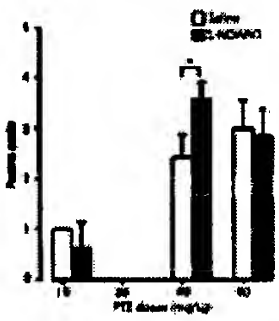

Figure 3 - Effect of L-NOARG treatment on PTZ-induced seizures. Note that potentiation of limbic seizures by L-NOARG treatment only occurred at the 60 mg/kg dose. Animals were evaluated for limbic seizures by the Racine scale (*$P<0.05$; **$P<0.01$; MANOVA). Note that 30 mg/kg PTZ elicited no limbic seizures (Racine scale = 0).

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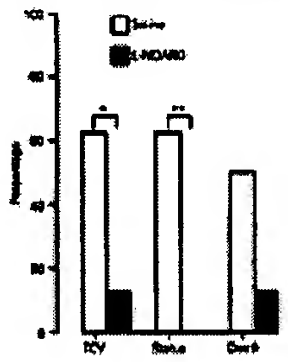

Figure 4 - Percentage of tonic convulsion (TCV), status epilepticus and fatalities after pretreatment with L-NOARG of PTZ (80 mg/kg)-injected animals (*P<0.05; **P<0.001; Fisher exact test). See also Figure 5.

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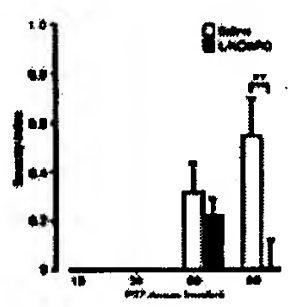

Figure 5 - Effect of L-NOARG pretreatment on the brainstem component of PTZ (80 mg/kg)-induced seizures. This severity index (SI) (30,34) includes tonic extensions (see Methods) as the end point of convulsive activity. Observe the clear protection against this tonic activity by L-NOARG (*P<0.05; **P<0.01; MANOVA). See also Figure 4. Note that 15 mg/kg and 30 mg/kg PTZ had no effect on tonic-clonic seizures (SI = 0).

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Discussion

The present data demonstrate that the effects of NOS inhibition and consequently the inhibition of NO synthesis on seizure activity depend on the seizure model (sensory or chemical) and, in the case of chemical induction, on the type and dose of the convulsant drug.

As far as we know, this is the first demonstration that the same dose of the NOS inhibitor L-NOARG can exert both anticonvulsant and proconvulsant effects on PTZ-evoked seizures. Thus, we suggest that seizure activity can be modulated by NO in different ways by means of either proconvulsant (PILO- and PTZ-evoked limbic seizures) or anticonvulsant (status epilepticus, tonic seizures and death evoked by the highest doses of PTZ) actions.

Limbic seizure potentiation was observed after acute L-NOARG treatment in the animals treated with the 100 mg/kg PILO dose and the 60 mg/kg PTZ dose. No effect of L-NOARG treatment was found with the 15 and 30 mg/kg dose of PTZ. Anticonvulsant L-NOARG effects were observed with PTZ-induced tonic seizures.

The doses of L-NOARG used in the present study were generally higher than those reported to protect against ischemic or excitotoxic lesions (5,36). The drug treatment regimen used in this study produced a substantial reduction of NO synthesis. It has been previously shown that a single injection of L-NOARG produces 50% inhibition and that four days of administration produce 95% inhibition of NO synthase (37,38). Moreover, drugs were given at doses that were previously shown to affect behavior (39-43) or seizure activity (9,44). These findings suggest

that the effect of L-NOARG on seizure models involves a decrease in NO formation in the CNS.

Furthermore, although the L-NOARG doses of the present experiments have been commonly employed in the literature, some studies have found inverted U-shaped dose-response curves for various NOS inhibitors (42). Therefore, it would be interesting to test additional doses of L-NOARG in the epilepsy models used in our study.

It is interesting to note that in the present study there was a difference between acute and chronic L-NOARG treatment. Acute L-NOARG treatment had a facilitatory effect on a subconvulsant PILO dose (100 mg/kg) for facial automatisms and status epilepticus. Additionally, salivation, piloerection and facial automatisms decreased significantly after chronic L-NOARG treatment. This is in agreement with recent data on catalepsy induced by acute L-NOARG treatment, although catalepsy was significantly decreased after chronic administration of the drug (42). These data contrast with other studies showing no difference between the acute and chronic effect of L-NOARG on the prolongation of bicuculline-induced seizures (13) and potentiation of kainic acid-induced seizures (8).

The protective effect of the acute L-NOARG treatment on the tonic seizures induced by 80 mg/kg PTZ is in agreement with data from Osonoe et al. (11) who examined the effect of systemic injection of L-NAME and L-NOARG on PTZ-induced seizures, reaching the conclusion that both drugs preferentially suppressed tonic generalized extension and prolonged its onset latency. Initiation and generalization of PTZ-induced seizures are related to the activation of NMDA receptors (45) and to competitive inhibition of GABA neurotransmission. GABA and NMDA increase intracellular Ca^{2+}, which activates NO synthase. Thus, L-NOARG inhibition of NO synthase could help suppress the tonic component of PTZ-induced seizures, protecting against their lethal effect. These results support the suggestion that NO contributes to the genesis of limbic seizure activity.

The effects of L-NOARG on limbic seizure models (PILO) and the tonic component of PTZ-induced seizures are in contrast to the lack of interference of L-NOARG with GEPR-3s audiogenic seizure, a model of brainstem-dependent seizures (29). Interestingly, a recent publication on kainate-induced behavioral and electrographic seizures in mice (46) demonstrated that L-NAME can display proconvulsant effects depending on the route of administration of the kainate (either systemic or intra-hippocampal). An intriguing fact is that L-NAME potentiated kainate-induced wild running, but not necessarily clonus (limbic pattern), suggesting the involvement of brainstem mechanisms.

The behavioral expression of audiogenic seizures, a model of genetically dependent generalized tonic-clonic seizures, seems to depend almost exclusively on brainstem substrates (29,30). Among other mechanisms, inferior colliculus NMDA neurotransmission plays a critical role in the expression of AS (47). Because there is a potent link between NMDA activity and NO synthesis (23), we expected to obtain some effects from the L-NOARG treatment of AS-resistant or -susceptible rats. Recently, however, Grassi et al. (48) have shown that, although microinfusion of L-NAME into the inferior colliculus was able to reduce middle latency auditory evoked responses (thalamo-cortical generators), the same treatment did not change the midbrain-evoked responses, particularly the collicular component (wave V). Additionally, Iannone et al. (49) also showed that L-NAME induced a blockade of electrocortical desynchronization, an effect which is compatible with telencephalic activity. Thus, experiments involving microinjection of either L-NOARG or L-NAME into the inferior colliculus are necessary in order to rule out NO participation in AS. The present data, based on L-NOARG

systemic treatment, did not show any clear involvement of NO in AS.

At the moment it is not possible to explain the molecular mechanisms responsible for the effect of L-NOARG on seizures. Acute effects of NO might involve an influence of NO on NMDA-mediated neurotransmission. NO has a complex influence on this neurotransmission. For example, it may mediate the NMDA-induced increase in cGMP but simultaneously inhibit the NMDA-induced increase in intracellular Ca^{2+} and NOS activity, and block NMDA receptors (20,23). The influence of NO on NMDA neurotransmission may vary widely according to drug concentration and site of injection, which might explain the range of conflicting results on the role of NO in NMDA-modulated events such as epilepsy, neurotoxicity, long-term potentiation and nociception (5,20, 50,51). For instance, in a very recent study Proctor et al. (52) demonstrated that exogenously applied NO or its precursors can enhance seizure-triggering activity. However, in an NMDA-dependent focally evoked seizure model (activation of the area tempestas), they demonstrated that the L-arginine-nitric oxide pathway does not normally contribute to seizure expression from the area tempestas.

This biphasic effect of NOS inhibitors is not unusual for drugs with anticonvulsant activity (53). Moreover, the dual role of NOS inhibitors was observed in studies of experimental anxiety (41), paradoxical results which we may extend to those obtained with NOS inhibitors in the current animal models of epileptic seizures.

The present experiments confirm that NO is clearly involved in seizure modulation through a complex set of mechanisms including both proconvulsant and anticonvulsant capabilities. Thus, our data suggest a dual role for NO in seizure modulation, which seems to be dependent on the epilepsy model and, in the case of drugs, on the dose actually used. An understanding of the molecular mechanisms of this dual active pro- and anticonvulsant effect depends on more specific protocols which we are currently developing in our laboratories.

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RELATED PROCEEDINGS APPENDIX

Appellant's representative are not aware of any related proceedings before the U.S. Patent and Trademark Office with respect to this patent application.